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Respectfully submitted,

Allen C. Turner
Registration No. 33,041
Attorney for Applicant(s)
TRASKBRITT
P.O. Box 2550
Salt Lake City, Utah 84110-2550
Telephone: 801-532-1922

Date: November 22, 2005
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Vlaams Interuniversitair Instituut voor
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Rijvisschestraat 120
9052 Zwijnaarde/BE

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
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Reversed mammalian protein-protein interaction trap

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# REVERSED MAMMALIAN PROTEIN-PROTEIN INTERACTION TRAP

The present invention relates to a recombinant receptor, comprising a ligand-binding domain and a signaling domain that comprises a heterologous bait polypeptide, which receptor is inactivated by binding of a prey polypeptide to said heterologous bait peptide, either in presence or absence of a ligand binding to said ligand-binding domain. The receptor is activated by addition of a compound that disrupts the bait-prey interaction. The present invention also relates to a method to screen compounds that disrupt compound-compound binding using said recombinant receptor.

Protein - protein interactions are an essential key in all biological processes, from the replication and expression of genes to the morphogenesis of organisms. Protein - protein interactions govern amongst others ligand - receptor interaction and the subsequent signaling pathway; they are important in assembly of enzyme subunits, in the formation of biological supramolecular structures such as ribosomes, filaments and virus particles and in antigen - antibody interactions.

Researchers have developed several approaches in attempts to identify protein - protein interactions. Co-purification of proteins and co-immunoprecipitation were amongst the first techniques used. However, these methods are tedious and do not allow high throughput screening. A major breakthrough was obtained by the introduction of the genetic approaches, of which the yeast two-hybrid (Fields and Song, 1989) is the most important one. Several improvements and modifications of this system have been published; as an example we can cite US5637463 that is describing a method to detect posttranslational modification dependent protein-protein interactions.

Approaches based on phage display do avoid the nuclear translocation. WO9002809 describes how a binding protein can be displayed on the surface of a genetic package, such as a filamentous phage, whereby the gene encoding the binding protein is packaged inside the phage. Phages, which bear the binding protein that recognizes the target molecule are isolated and amplified. Several improvements of the phage display approach have been proposed, as described e.g. in WO9220791, WO9710330 and WO9732017.

Another technique for assessing protein-protein interactions is based on fluorescence resonance energy transfer (FRET) or bioluminescence resonance energy transfer (BRET; WO9966324).

Although in principle, all those methods can be used in a positive (detecting protein-protein interactions) or a negative (detection of disruption of the protein-protein interaction) way, due to the positive selection in case of protein-protein interaction, none of these methods is really suited for screening of compounds that inhibit protein-protein interaction. To overcome this problem, US5733726 discloses a cytotoxicity-based genetic selection method, whereby a classic two-hybrid system is linked to the transcriptional activation of a toxic reporter gene, providing an assay for positive selection of mutations or small molecules or drugs disruptive for protein-protein interaction. An adaptation of this method has been disclosed in WO9813502, whereby the protein-protein interaction induces the transcription of a repressor gene, which represses a selectable marker. Upon disruption of the protein-protein interaction, the repressor will not longer by synthesized, resulting in an activation of the selectable marker.

There is, however, still a need for a selection system for compounds that disrupt protein protein interaction, which can study this disruption under physiological conditions, with a low and controllable background.

The present invention provides a method to screen compounds that disrupt compound-compound interaction, which satisfies this need and provides additional advantages as well. The principle of the method is illustrated in Figure 1.

It is one aspect of the invention to provide a recombinant transmembrane receptor, comprising an extracellular ligand binding domain and a cytoplasmic domain that comprises a heterologous bait polypeptide, whereby the activation of said receptor is inhibited by binding of a prey polypeptide to said heterologous bait peptide. The recombinant receptor can be a chimeric receptor, in which the ligand binding domain and the cytoplasmic domain are derived from two different receptors. Preferably, the receptor is a multimerizing receptor; this can be a homomultimerizing receptor as well as a heteromultimerizing receptor. The cytoplasmic domain of the recombinant receptor comprises a heterologous bait polypeptide, which can be fused to the carboxyterminal end, or can replace a part of this carboxyterminal end or can be situated in the cytoplasmic domain itself, as an insertion or a replacement of an endogenous internal fragment. In case of a heteromultimerizing receptor, not all the chains need to comprise the bait, but it is sufficient if one of the composing chains does comprise the bait in its cytoplasmic domain. Insertion of the heterologous bait polypeptide may result in one or more deletions of the original cytoplasmic domain. The only limiting factor for the

modifications in the cytoplasmic domain is that said cytoplasmic domain should retain, directly or indirectly, its inherent modifying enzyme activity, either by retaining a modifying enzyme activity binding site such as a Jak binding site, or by incorporating an active modifying enzyme activity in the cytoplasmic domain itself, and that said cytoplasmic domain should retain, directly or indirectly, at least one activation site. This activation site is not necessarily situated on the cytoplasmic domain itself, but may be situated on another polypeptide that binds, directly or indirectly, to the cytoplasmic domain. Activation of the receptor and of the signaling pathway is achieved by binding of a ligand to the ligand-binding domain of the receptor, and by disruption of the binding of a prey polypeptide to the heterologous bait polypeptide comprised in the cytoplasmic domain of the receptor. Alternatively, a constitutively activated (i.e. non-ligand dependent) receptor may be used, whereby the activation is inhibited by binding of a prey polypeptide to the heterologous bait polypeptide comprised in the cytoplasmic domain of the receptor, which then may solely be activated by disruption of said binding. One preferred embodiment is a recombinant receptor according to the invention whereby the activation site is a phosphorylation site and the modifying enzyme activity is a kinase.

Another preferred embodiment of the invention is a homomultimerizing recombinant leptin receptor, with a heterologous bait polypeptide fused into, or, preferentially, at the carboxyterminal end of its cytoplasmic domain. Said heterologous bait polypeptide may replace part of said cytoplasmic domain, as long as the Jak binding site and one activation site are retained.

It is another aspect of the invention to provide a recombinant receptor, comprising an extracellular ligand binding domain and a cytoplasmic domain that comprises a heterologous bait polypeptide which can be modified by modifications such as, but not limited to phosphorylation, acetylation, acylation, methylation, ubiquitinilation, glycosylation or proteolytic processing, whereby said recombinant receptor is activated by binding of a ligand to said ligand binding domain and by disruption of the binding of a prey polypeptide to said heterologous bait polypeptide and whereby said binding of the prey polypeptide to the heterologous bait polypeptide is dependent upon the modification state of the heterologous bait polypeptide, i.e. either there is only, or preferentially, binding with modification, or there is only, or preferentially, binding without modification. Said modification state can be, as a non-limiting example, the presence or absence of phosphorylation, acetylation, acylation, methylation,

ubiquitinilation or glycosylation, or occurrence of proteolytic cleavage or not. The recombinant receptor can be a chimeric receptor, in which the ligand binding domain and the cytoplasmic domain are derived from two different receptors. Preferentially, the receptor is a multimerizing receptor. As described above, the cytoplasmic domain of the recombinant receptor comprises a heterologous bait polypeptide, which can be fused to the carboxyterminal end, or can replace a part of this carboxyterminal end or can be situated in the cytoplasmic domain itself, as an insertion or a replacement of an endogenous internal fragment. In case of a heteromultimerizing receptor, not all the chains need to comprise the bait, but it is sufficient if one of the composing chains does comprise the bait in its cytoplasmic domain.

The modification of the bait may be either in cis or in trans, i.e. by an enzymatic activity that is situated on the same cytoplasmic domain, or by an enzymatic activity that is not linked to the receptor such as an enzyme that circulates freely in the cytosol. Preferentially, the modification of the bait is induced by binding of a ligand to the ligand-binding domain. One preferred embodiment is a homodimerizing receptor in which the bait is phosphorylated by the inherent kinase activity of the cytoplasmic domain, preferentially a Jak kinase that is binding to said cytoplasmic domain. Another preferred embodiment is a heteromultimerizing receptor where the cytoplasmic domain of one chain comprises a bait to be modified, and the cytoplasmic domain of another chain comprises the modifying activity.

Activation of the receptor and of the signaling pathway is achieved by binding of a ligand to the ligand-binding domain and by disruption of the binding of a prey polypeptide to the heterologous bait polypeptide situated in the cytoplasmic domain of the receptor. Preferentially, binding of said prey polypeptide is dependent upon the modification state of said heterologous bait polypeptide, it means that binding occurs only in case the bait is modified or only in case the bait is not modified.

It is another aspect of the invention to provide a prey polypeptide, whereby said prey polypeptide is a fusion protein comprising a polypeptide that can interact directly or indirectly with a bait polypeptide and another polypeptide that comprises an inhibitor of the activation of the receptor and/or a recruitment site for an inhibitor of the activation of the receptor. Said inhibitor is preferentially an inhibitor of phosphorylation, more preferentially an inhibitor of tyrosine phosphorylation. One preferred embodiment is a prey polypeptide, whereby said inhibitor is a SH2-containing protein tyrosine phosphatase (SHP), or a functional part thereof. Another preferred embodiment is a

prey polypeptide, whereby said inhibitor is a member of the Suppressor Of Cytokine Signaling (SOCS) family, or a functional part thereof. Most preferentially, said inhibitor is SOCS1 or SOCS3, or a functional part thereof. Direct interaction means that there is a direct protein-protein contact between the heterologous bait polypeptide and the prey polypeptide; indirect interaction means that the heterologous bait polypeptide interacts with one or more other polypeptides to form a complex that interacts with said prey polypeptide or vice versa. In the latter case, the prey polypeptide may interact either with only one or with several polypeptides from the complex. The binding of the prey polypeptide to the bait polypeptide may be dependent upon the modification state of said bait polypeptide and/or of proteins within the binding complex.

In case that disruptions of interactions of nuclear proteins are studied, the prey polypeptide may comprise a Nuclear Export Sequence (NES), to ensure that it is available in the cytosol. The NES signal (amino acids 37-46) of the heat stable inhibitor of the cAMP-dependent protein kinase has been shown to override a strong nuclear localization signal (Wiley *et al.*, 1999). This NES will keep the prey polypeptide in the cytoplasm even if it has a strong nuclear localization signal, facilitating the interaction with the bait.

One preferred embodiment is a prey polypeptide according to the invention, whereby said prey polypeptide interacts with the heterologous bait polypeptide of a recombinant receptor according to the invention. Upon binding of a ligand to the ligand binding domain and upon direct or indirect interaction of said heterologous bait polypeptide with said prey polypeptide, the inhibitor of the prey polypeptide will inhibit the modification of the activation site by modifying enzyme activity inherent to the cytoplasmic domain of the receptor, and so inhibit the activation of the receptor. Preferentially, said inhibitor is an inhibitor of phosphorylation, said activation site is a phosphorylation site and the modifying enzyme activity is a kinase activity. More preferentially, the activation of the receptor comprises binding of a STAT polypeptide to the phosphorylated phosphorylation site, followed by phosphorylation of said STAT polypeptide and subsequent dimerization of two phosphorylated STAT molecules.

Another aspect of the invention is a vector, encoding a recombinant receptor according to the invention and/or a vector, encoding a prey polypeptide according to the invention. Said recombinant receptor and said prey polypeptide may be situated on one or on separated vectors. The vector can be any vector, know to the person skilled in the art, including but not limited to episomal vectors, integrative vectors and viral vectors.

Another aspect of the invention is a eukaryotic cell comprising a recombinant receptor according to the invention. Preferentially, the eukaryotic cell is obtained by transformation or transfection with one or more vectors according to the invention. Said eukaryotic cell comprises, but is not limited to yeast cells, fungal cells, plant cells, insect cells and mammalian cells. Preferentially, the eukaryotic cell is a mammalian cell.

Still another aspect of the invention is a kit, comprising one or more cloning vectors allowing the construction of one or more vectors according to the invention. It is clear for the people skilled in the art that a cloning vector, encoding a recombinant receptor in which the part, encoding for the cytoplasmic domain comprises one or more restriction sites allowing an "in frame" fusion of a nucleic acid fragment encoding a polypeptide can easily be used to construct a vector encoding a recombinant receptor according to the invention. In a similar way, a cloning vector encoding a first polypeptide comprising at least one inhibiting domain and/or a recruitment site for an inhibitor, such as a SOCS or SHP recruitment site, comprising one or more restriction sites allowing an "in frame" fusion of a nucleic acid encoding a second polypeptide with said first polypeptide can easily be used to construct a vector encoding a prey polypeptide according to the invention. Alternatively, both for the construction of the vector encoding the recombinant receptor and for the vector encoding the prey polypeptide, other cloning strategies known to the person skilled in the art may be used.

Still another aspect of the invention is a method to screen compounds disrupting compound-compound binding using a recombinant receptor and/or a prey polypeptide according to the invention. In a preferred embodiment, a eukaryotic cell, carrying a recombinant receptor according to the invention and a prey polypeptide according to the invention is treated with a compound library. Disruption of bait-prey binding will result in an activation of the signaling pathway and can be detected by the use of a reporter system. Said compound library may be added as extracellular compounds the cell, or may be produced within said cell, as peptides or polypeptides, possibly after transformation or transfection of said cell with a library encoding said peptides or polypeptides.

One specific embodiment of the method to screen compounds disrupting compound-compound binding is a method whereby said binding is a protein-protein interaction. Another specific embodiment is a method to screen compounds disrupting protein-protein interaction, whereby said interaction is modification state dependent. Said compounds may be as a non-limiting example, an inhibitor of the modification in case

of a modification dependent protein-protein interaction of a bait modifying enzyme in case where the protein-protein interaction occurs only, or preferentially in absence of modification. Still another specific embodiment is a method to screen compounds disrupting compound-compound binding, whereby said binding is mediated by three or more partners. In this case, one or more partners may not be or not completely be of proteineous nature. It is clear for a person skilled in the art that a recombinant receptor, according to the invention may, as a non-limiting example, bind to a small molecule. On the other hand, the prey polypeptide, according to the invention may also bind to the small molecule, so that bait and prey are linked together by said small molecule. Said small molecule may be present in the host cell, as a compound produced by the cell itself, or as a compound that is taken up from the medium.

Preferably, said method to screen compounds that disrupt compound-compound binding comprises the construction of a eukaryotic cell comprising a recombinant receptor according to the invention and a prey polypeptide according to the invention. Said cell is brought in contact with a compound library, that are added as extracellular compounds and taken up by the cell, or that are produced within the cell, after transformation of said cell with a DNA library encoding said compounds. The disruption of the compound-compound binding is detected by the activation of the receptor, leading to an active signaling pathway, resulting in the induction of a reporter system. A reporter system can be any system that allows the detection and/or the selection of the cells carrying a recombinant receptor according to the invention. It is clear for the person skilled in the art that several reporter systems can be used. As a non-limiting example, a luciferase gene, an antibiotic resistance gene or a cell surface marker gene can be placed after a promoter that is induced by the signaling pathway. Alternatively, reporter systems may be used that are based on the change in characteristics of compounds of the signaling pathway, when said pathway is active, such as the phosphorylation and/or dimerization of such compounds.

**Definitions**

The following definitions are set forth to illustrate and define the meaning and scope of various terms used to describe the invention herein.

*Receptor* as used here does not necessarily indicate a single polypeptide, but may indicate a receptor complex, consisting of two or more polypeptides, and comprising a ligand binding domain and a cytoplasmic domain. Recombinant receptor means that at

least one of said polypeptides is recombinant. Preferably the polypeptide comprising the cytoplasmic domain is recombinant. *Cytoplasmic domain* as used here includes the transmembrane domain.

*Inhibition of activation* of a receptor by binding of a prey polypeptide to a heterologous bait peptide is every action by which a receptor that is activated in absence of said binding shows a reduced induction of its signaling pathway, as measured by the reduced output of the reporter system, independent of the mechanism used. This can be, as a non-limiting example, by inhibition of the modifying enzyme activity, by blocking of the activation site, or by inhibition of binding of one of the compounds of the signaling pathway. Reduced induction is any reduction of induction that can be measured in a reliable way, by comparing the situations of binding and absence of binding. Preferentially, the induction of signaling is ligand dependent. However, alternatively, a ligand-independent constitutive signaling receptor may be used.

*Reporter system* as used here may be any system, known to the person skilled in the art that results in a detectable output upon activation of the receptor. As some non-limiting examples, cell survival or luciferase activity may be used.

*Activation site* of a receptor is the site that, in the wild type receptor, is modified after binding of a ligand to the ligand binding domain, leading to a clustering and/or reorganization of the receptor and subsequent activation of the modifying enzyme activity, and to which a compound of the signaling pathway can bind after modification, or any site that can fulfill a similar function. In the latter case, the activation site is not necessarily located on the same polypeptide as in the wild type receptor, but may be situated on another polypeptide of the receptor complex.

*Modifying enzyme activity* as used here means the enzymatic activity, associated to or incorporated in the cytoplasmic domain of the receptor that is induced upon binding of the ligand to the ligand binding domain and subsequent reorganization of the receptor (e.g. by a conformational change), and may modify the activation site. Preferably, the activation site is a phosphorylation site and the modifying enzyme activity is a kinase activity

*Activation of a receptor* as used here means that the receptor is inducing a signaling pathway, by binding of a compound of the signaling pathway to the modified activation site, whereby said activation normally results in the induction or repression of one or more genes. Said gene is preferentially a reporter gene, which allows monitoring the activation of the receptor. An *activated receptor* is a receptor where the binding of a

compound to the activation site has been enabled by modification of said site.

*Multimerizing receptor* as used here means that the activated receptor comprises several polypeptides. It does not necessarily imply that the multimerization is induced by ligand binding: the receptor can exist as a preformed complex of which the conformation is changed upon ligand binding.

*Polypeptide* as used here means any proteineous structure, independent of the length and includes molecules such as peptides, phosphorylated proteins and glycosylated proteins. Polypeptide as used herein is not necessarily indicating an independent compound but can also be used to indicate a part of a bigger compound, such as a domain of a protein.

*Heterologous bait polypeptide*, as comprised in the receptor means that within the receptor, or fused to the receptor, but not in the ligand-binding domain of said receptor, there is a polypeptide that is not present in the non-recombinant receptor. In case of a transmembrane receptor, the heterologous bait is fused within the cytoplasmic domain, or fused to the cytoplasmic domain. Said heterologous bait may replace a part of said cytoplasmic domain. Bait herein means that this polypeptide can interact with other polypeptides, not belonging to the normal receptor complex.

*Prey polypeptide* as used here means a fusion protein comprising a polypeptide that can bind with the heterologous bait polypeptide and a polypeptide that comprises an inhibitor of the receptor and/or a recruitment site for an inhibitor of the receptor, i.e every polypeptide that can directly or indirectly inhibit the activation the receptor according to the definition above.

*Ligand* means every compound that can bind to the ligand binding domain of a receptor and that is able to initiate the signaling pathway by binding to said ligand-binding domain. Initiating as used here means starting the events that follow normally directly the binding of the ligand to the ligand-binding domain of a receptor, e.g. multimerization for a multimerizing receptor, but it does not imply activation of the receptor and/or accomplishing of the signaling pathway.

*Functional part of an inhibitor*, such as a functional part of SHP or SOCS means any part that still can fulfill its inhibiting activity. In case of a protein phosphatase, it is a part that still shows protein phosphatase activity.

*Compound* means any chemical or biological compound, including simple or complex organic or inorganic molecules, peptides, peptido-mimetics, proteins, antibodies, carbohydrates, nucleic acids or derivatives thereof.

*Bind(ing)* means any interaction, be it direct or indirect. A direct interaction implies a contact between the binding partners. An indirect interaction means any interaction whereby the interaction partners interact in a complex of more than two compounds. This interaction can be completely indirect, with the help of one or more bridging compounds, or partly indirect, where there is still a direct contact that is stabilized by the interaction of one or more compounds.

*Cloning vector* is a vector that is generally considered as an intermediate step for the construction of another vector. It is intended to insert one or more nucleic acid fragments, in order to obtain one or more new vectors that will be used to transform the host cell of interest.

**Brief description of the figures**

Figure 1: Principle of the reversed mammalian protein-protein interaction trap. B: bait; K: modifying enzyme activity; L: ligand; M: possible modification of the bait; P: prey. F and Y represent the amino acids that function as a mutated (F) or functional (Y) receptor activation site, or inhibitor recruitment site. The example illustrates a tyrosine phosphorylation site. As is shown in the figure, the bait-prey interaction may be modification dependent. The inhibitor, such as SOCS or a functional part thereof, is fused to the prey polypeptide, as illustrated at the left hand side. Alternatively, an inhibitor recruitment domain, such as a SOCS or SHP recruitment domain may be fused to the prey, as illustrated at the right hand side. Both domains may be connected by a hinge region, such as a GGS sequence, to optimize the flexibility of the protein and the resulting negative feedback. The prey polypeptide may be fused to a "flag"-sequence, to facilitate identification and/or isolation, but this is not an essential feature.

Figure 2: Specific inhibition of activation of the EpoR-LepRFFY-EpoR by the SOCS3 CISSH2 chimera is disrupted by overexpression of SOCS2.

**Examples**

***Materials and methods to the examples***

*Cell lines and transfection procedure*

Transfections were performed according to the calcium phosphate method (Graham and van der Eb, 1973).

Recombinant human erythropoietin (Epo) was purchased from R&D Systems. Typical stimulation conditions were 50 ng/ml Epo.

HEK293T cells were maintained in a 10 % $CO_2$ humidified atmosphere at 37°C, and were grown using DMEM with 4500 mg/ml glucose, 10 % fetal bovine serum and 50 µg/ml gentamycin (all from LifeTechnologies)

*Construction of the plasmids*

- *Generation of the pSEL1*

The mutant leptin receptors (Eyckerman *et al.*, 1999) Y985-1077F and Y985-1077-1138F (LepR-F3) were generated using the Quikchange™ site-directed mutagenesis procedure using Pfu polymerase (Stratagene) on the pMET7-LepR template. Mutagenic oligonucleotides were MBU-O-157, MBU-O 158, MBU-O-159, MBU-O-160, MBU-O-161 and MBU-O 162. Each single mutation was coupled to a change in restriction cleavage and was confirmed by restriction and DNA sequence analysis. The double and triple mutants were created using a sequential approach. PCR amplification on this pMET7-LepR-F3 vector template using MBU-O-447 and MBU-O-448 as forward and reverse primers, respectively, resulted in a LepR-F3 amplicon spanning the transmembrane and intracellular domains of LepR-F3 (+1 extra Gly of the extracellular part), which was subcloned in the pCR®-Blunt vector (Invitrogen). PacI-SacI digestion of the resulting plasmid yielded a DNA fragment containing the LepR-F3 sequence, which was ligated into PacI-SacI digested and gel-purified pSV-SPORT-EpoR/IFNaR2-2 vector (Pattyn *et al.*, 1999). This resulted in the pSV-SPORT-EpoR/LepR-F3, which was renamed to pSEL1.

- *Construction of pSEL1-EpoR*

RNA was prepared from $5x10^6$ TF-1 cells using the RNeasy kit (Qiagen), and eluted in 50 µl water from which 10 µl was used as input for RT-PCR. Standard RT-PCR was performed as follows: 2 µl (2µg) of oligodT (12-18 mer; Pharmacia) was added and incubated at 70°C for 10 min., the reaction mixture was chilled on ice for 1 min., cDNA was prepared by adding 4 µl of 10x RT buffer (Life Sciences), 1µl 20 mM dNTP's (Pharmacia), 2µl 0,1M DTT, and 1µl of MMLV reverse transcriptase (200U; Superscript RT; Life Technologies) to an end volume of 20µl. Incubations were as follows: RT for 10 min., 42°C for 50 min., 90°C for 5 min., and 0°C for 10 min. Following this, 0,5 µl RnaseH (2U; Life Technologies) was added and the mixture was incubated at 37°C for 20 min., followed by chilling on ice. PCR on this cDNA was performed using Pfu

enzyme (5 U; Stratagene). An intracellular fragment of the human EpoR (amino acids 370-453) was amplified from 4µl TH-1 cDNA using MBU-O-675 and MBU O-676 as forward and reverse primer respectively, with two consecutive PCR reactions and an intermediate gel-purification. SacI and XbaI recognition sites are present in the forward and reverse primers respectively. The reverse primer also encodes a stop codon. After gel-purification of the PCR amplicon band of the correct size, the fragment was subcloned in pCR®-Blunt, digested with SstI (which has the same recognition site as SacI) and XbaI, and ligated into SstI-XbaI digested and gel-purified pSEL1 vector, resulting in the pSEL1-EpoR construct.

- *Construction of pSV-EpoR-LepRFFY-EpoR*

The pSV-EpoR-LepRTTY-EpoR was generated by amplifying the transmembrane and the intracellular part of the murine leptin receptor containing the Y985F and the Y1077F mutations by using primers MBU-O-447 and MBU-O-448 and the pMET7-LepRFFY construct (Eyckerman et al., 1999) as template. The forward primer contains a PacI site allowing in frame fusion with the extracellular part of the erythropoietin receptor. The reverse primer contains SalI, SstI, NotI and XbaI sites. The LepRFFY fragment was cloned in pSEL1-EpoR via a PacI-SstI based exchange, preceded by a subcloning step in pCR-Blunt (Invitrogen). This leads to pSV-EpoR-LepRFFY-EpoR.

- *Construction of pMET7-flag-gp13*

The pMET7mcs vector is a modified version of pMET7 (Takabe et al, 1988) containing an expanded MCS by insertion of the extra unique BglII, EcoRV, DstEII, AgeI and XhoI restriction sites. PCR amplification on the pSVL-gp130 template using the forward primer MBU-O-586 and the reverse primer MBU-O-443 generated a DNA fragment encoding a 158 amino acid-long intracellular fragment of the human gp130 chain, which contains 4 STAT-3 association motifs (amino acids 761-918, the stopcodon was not co-amplified). The forward primer contains from 5' to 3' an ApaI restriction site, a Kozak consensus sequence, a flag-tag encoding sequence (Met-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-Ile), and a BglII restriction site. The reverse primer encodes an additional hinge sequence (Gly Gly-Ser) and contains an EcoRI recognition site. ApaI and EcoRI digestion of the PCR product (after subcloning in pCR-Blunt) and of pMET7-mcsA, allowed us to ligate the gp130 fragment into the pMET7 vector, generating the pMET7-flag-gp130 construct.

- *Construction of pMG1-SVT*

SV40 largeT antigen (SVT) was amplified using a vector from the HybriZAP 2.1 Two-Hybrid cDNA synthesis kit (Stratagene, pSV40) as template. Primers MBU-O-445 and MBU-O-446 were used to generate a DNA fragment encoding 448 amino acid between residues 261 and 708. The N-terminal deletion eliminates the nuclear targeting signal in SVT. The forward primer contains an EcoRI recognition site that allows in-frame ligation to the gp130-hinge sequence. The reverse primer contains additional NruI, XhoI, BglII, NotI and XbaI restriction sites and also encodes the stop codon after the SVT coding sequence. Subcloning in pCR®-Blunt, followed by recovery of the cleaved amplicon with EcoRI and XbaI, allowed ligation in the EcoRI-XbaI opened pMET7-flag-gp130 vector, yielding pMET7-flag-gp130-SVT, which was renamed to pMG1-SVT.

- *Construction of pMG1-CIS*

The complete coding region for mouse Cytokine Inducible SH2-containing protein CIS (amino acids 2-257) was amplified using MBU-O-677 and MBU-O-678 as forward and reverse primer respectively. The forward primer contains an EcoRI recognition site and the reverse primer contains an XbaI recognition site and the stop codon. The amplified and gel-purified fragment was subcloned into the pCR®-Blunt vector (Invitrogen). The insert was recovered by EcoRI and XbaI digestion and gel-purification and was cloned into an EcoRI-XbaI digested and gel-purified pMG1-SVT vector, leading to the pMG1-CIS vector.

- *Construction of pMET7-fSOCS3.*

Prey constructs were generated in the pMET7 vector, which contains the strong constitutive SRα promotor (Takebe et al., 1988). Rat SOCS3 cDNA was amplified using MBU-O-302 and MBU-O-303 as forward and reverse primer respectively, and using mRNA from leptin-stimulated PC12 cells (rat pheochromocytoma cell line) as template. cDNA was prepared using a standard RT procedure with Superscript Reverse Transcriptase (LifeTechnologies). Amplification was performed using Pfu polymerase (Stratagene). The complete SOCS3 coding fragment was reamplified using forward primer MBU-O-837 and the reverse primer described above, which allows BglII-XbaI based cloning in pMG1-CIS, resulting in an expression vector wherein the complete coding sequence of SOCS3 is N-terminally fused to a FLAG tag sequence (MDYKDDDDK). This construct was named pMET7-fSOCS3.

- *Construction of pMET7-fSOCS3 CISSH2*

Unique BspEI and XhoI sites were created by site directed mutagenesis (Quikchange™ Site Directed Mutagenesis Kit, Stratagene) in both the SOCS3 construct and the pMG1-CIS construct, while respecting the amino acid sequence. The BspEI site was created in front of the SH2 domain, while the XhoI site was inserted in front of the SOCS box sequence. Mutagenic primers for BspEI in SOCS3 were MBU-O-1005 and MBU-O-1006 as forward and reverse respectively. Primers for insertion of the XhoI site in SOCS3 were MBU-O-1007 and MBU-O-1008. BspEI was created in pMG1-CIS using MBU-O-1001 and MBU-O-1002, while XhoI was created using MBU-O-1003 and MBU-O-1004. The SOCS3 SH2 domain (amino acids 46-184) was swapped with the SH2 domain of CIS (amino acids 82-218), using a BspEI-XhoI based exchange. This construct was named pMET7-fSOCS3 CISSH2.

The pMET7-fCIS construct was generated by cutting the pEF-FLAG-I/mCIS construct (obtained from N. Nicola) with EcoRI-and PvuII, and the pMG1-CIS construct with PvuII-KpnI. This results in two fragments of the CIS cDNA, which were simultaneously cloned in a three-fragment ligation step in the EcoRI-KpnI opened pMET7mcs construct. The pEF-FLAG-I/SOCS2 construct expressing N-terminally flag-tagged full size murine SOCS2 was obtained from N. Nicola.

- *Other constructions*

Prey constructs were generated in the pMET7 vector, which contains a strong constitutive hybrid SRα promoter (Takebe *et al.*, 1988).

The pMET7mcs vector is a modified version of pMET7 containing an expanded MCS by insertion of the extra unique BglII, EcoRV, BstEII, AgeI and XhoI restriction sites.

The pUT651 construct expressing β-galactosidase was obtained from Eurogentec.

Generation of the pGL3-rPAP1-luci construct was described before (Eyckerman et al., 1999). The full-length rPAP1 promoter fragment was excised using partial digestion with KpnI and XhoI and ligated into the KpnI-XhoI digested pXP2d2 vector (gift from Prof. S. Nordeen), resulting in the leptin-responsive pXP2d2-rPAP1-luci reporter construct. The pXP2d2 vector is a derivative of pXP2 that lacks potential cryptic Activator Protein 1 sites (Grimm and Nordeen, 1999). All constructs were verified by restriction and sequence analysis.

*Example1: Specific inhibition of activation of the EpoR-LepRFFY-EpoR by the SOCS3 CISSH2 chimera is disrupted by overexpression of SOCS2*

Following combinations of plasmids were transfected in $4.10^5$ HEK293T cells.

a. pSV-EpoR-LepR FFY EpoR + pMET7mcs + pXP2d2-rPAP1-luci + pUT651

b. pSV-EpoR-LepR FFY-EpoR + pMET7-fSOCS3 CISSH2 + pXP2d2-rPAP1-luci + pUT651

c. pSV-EpoR-LepR FFY-EpoR + pMET7-fSOCS3 CISSH2 + pEF-FLAG-fSOCS2 + pXP2d2-rPAP1-luci + pUT651

d. pSV-EpoR-LepR FFY-EpoR + pMET7-fCIS + pXP2d2-rPAP1-luci + pUT651

DNA amounts in a 300 µl precipitation mixture were: 1µg pSV-EpoR-LepRFFY-EpoR chimeric receptor construct, 1µg of pXP2d2-rPAP1-luci reporter construct, 25 ng of pUT651 for normalization and 100 ng of the other plasmids. Additional pMET7mcs vector was added to keep DNA amounts constant in the transfections. 200 µl of this precipitation mixture was added to $4.10^5$ HEK293T cells in 6 well plates. 24 hours after transfection, cells were resuspended using Cell Dissociation Agent (LifeTechnologies) and seeded in black well plates. The seeded cells were stimulated for 24 hours with erythropoietin, or were left unstimulated. Reporter activity was measured using a luciferase assay and a TopCount chemiluminiscence counter (Canberra Packard). Results are shown in figure 2.

These results clearly show strong induction when the EpoR-LepRFFY-EpoR chimera is expressed. The induction is moderately inhibited by co-expression of the CIS wild type protein, but shows very strong inhibition upon co-transfection of the chimeric SOCS3 CISSH2 protein. The SH2 domain targets the SOCS3 inhibitory regions towards the activated complex, resulting in specific inhibition. When the SOCS2 protein is co expressed with the SOCS3 CISSH2 protein, inhibition is lost due to competition for the binding site, resulting in strong induction of the reporter gene.

Table 1: primers used for the constructions.

| | | |
|---|---|---|
| MBU-O-302 | forward mSOCS3 primer | 5' GAAGATCTCTCGCCATGGTCACCCACAGC AAGTT |
| MBU-O-303 | reverse mSOCS3 primer | 5' GCTCTAGATTTTGCTCCTTAAAGTGGAGCATC ATA |
| MBU-O-447 | forward mLepR primer | 5' GCTTAATTAACGGGCTGTATGTCATTGTACC |

| | | | |
|---|---|---|---|
| MBU-O-448 | reverse mLepR primer | 5' CGTCTAGATTAGCGGCCGCTTACTAGTGAGC TCGTCGACCCACCCACAGTTAAGTCACACATC | |
| MBU-O-837 | forward mSOCS3 primer | 5' GCGAGATCTCAGAATTCGTCACCCACAGCA AGTTTCC | |
| MBU-O-1001 | mutagenesis BspEI CIS | 5' CTCCTACCTTCGGGAATCCGGATGGTACTG GGGTTC | |
| MBU-O-1002 | mutagenesis BspEI CIS | 5' GAACCCCAGTACCATCCGGATTCCCGAAGG TAGCAC | |
| MBU-O-1003 | mutagenesis XhoI CIS | 5' CAGCCCTTTGTGCGCCGCTCGAGTGCCCGC A GCTTAC | |
| MBU-O-1004 | mutagenesis XhoI CIS | 5' GTAAGCTGCGGGCACTCGAGCGGCGCACAA AGGGCTG | |
| MBU-O-1005 | mutagenesis BspEI SOCS3 | 5' CCCAAGCTGCAGGAGTCCGGATTCTACTGG AGTGCC | |
| MBU-O-1006 | mutagenesis BspEI SOCS3 | 5' GGCACTCCAGTAGAATCCGGACTCCTGCAG CTTGCG | |
| MBU-O-1007 | mutagenesis XhoI SOCS3 | 5' GAGCCGACCTCTCTCGACCAACGTGGCTAC CCTC | |
| MBU-O-1008 | mutagenesis XhoI SOCS3 | 5' GAGGGTAGCCACGTTGCTCGAGAGAGGTCG GCTC | |
| MBU-O-157 | Y985F mutagenesis in mLepR | F | GAGACAACCCTCAGTTAAATTTGCAA CTCTGGTCAGCAACG |
| MBU-O-158 | Y985F mutagenesis in mLepR | R | CGTTGCTGACCAGAGTTGCAAATTTA ACTGAGGGTTGTCTC |
| MBU-O-159 | Y1077F mutagenesis in mLepR | F | GGGAGAAGTCTGTCTGTTTTCTAGGG GTCACCTCCGTCAAC |
| MBU-O-160 | Y1077F mutagenesis in mLepR | R | GTTGACGGAGGTGACCCCTAGAAAAC AGACAGACTTCTCCC |
| MBU-O-161 | Y1138F mutagenesis in mLepR | F | CTGGTGAGAACTTTGTACCTTTTATGC CCCAATTTCAAACCTG |
| MBU-O-162 | Y1138F mutagenesis in mLepR | R | CAGGTTTGAAATTGCCGCATAAAAGG TACAAAGTTCTCACCAG |
| MBU-O-443 | hgp130 primer | R | GCGAATTCCGAACCGCCCTGAGGCAT |

| | | | |
|---|---|---|---|
| | | | GTAGCCGCC |
| MBU-O-445 | SV40LargeT primer | F | GCGAATTCGAAGCAGAGGAAACTAAA CAAGTG |
| MBU-O-446 | SV40LargeT primer | R | CGTCTAGAGCGGCCGCAGATCTCGA GTCGCGATTATGTTTCAGGTTCAGGG GGAG |
| MBU-O-586 | hgp130 primer | F | GACGGGCCCGCCACCATGGATTACAA GGATGACGACGATAAGATCTCGACCG TGGTACACAGTGGC |
| MBU-O-675 | hEpoR intr. fragment primer | F | GGCGAGCTCGGTGCTGGACAAATGG TTGC |
| MBU-O-676 | hEpoR intr. fragment primer | R | CCCTCTAGATTACTTTAGGTGGGGTG GGGTAG |
| MBU-O 677 | mCIS primer | F | GCGGAATTCGTCCTCTGCGTACAGGG ATC |
| MBU-O-678 | mCIS primer | R | GCCTCTAGATCAGAGTTCGAAGGGGT ACTG |

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**Claims**

1. A recombinant receptor, comprising a ligand binding domain and a domain that comprises a heterologous bait polypeptide, whereby the activation of said receptor is inhibited by binding of a prey polypeptide to said heterologous bait peptide.

2. A recombinant receptor according to claim 1, whereby said receptor is a transmembrane receptor.

3. A recombinant receptor according to claim 1 or 2, whereby said receptor is activated by the addition of a compound that disrupt the bait-prey interaction.

4. A recombinant receptor according to any of the claims 1-3 whereby said receptor is a homomultimerizing receptor.

5. A recombinant receptor according to any of the claims 1-3 whereby said receptor is a heteromultimerizing receptor.

6. A recombinant receptor according to any of the claims 1-5 whereby the binding of said prey polypeptide is depending upon the modification state of said heterologous bait peptide.

7. A recombinant receptor according to claim 6 whereby said modification state is presence or absence of phosphorylation, acetylation, acylation, methylation, ubiquitinilation or glycosylation.

8. A recombinant receptor according to any of the claims 6-7 whereby the change of said modification state is dependent upon binding of a ligand to the ligand-binding domain.

9. A prey polypeptide, comprising a polypeptide that interacts with a bait polypeptide and a polypeptide comprising an inhibitor of activation of a receptor and/or a recruitment site for an inhibitor of activation of a receptor.

10. A prey polypeptide according to claim 9, comprising a polypeptide that interacts with the heterologous bait polypeptide of a recombinant receptor according to any of the claims 1-8 and a polypeptide comprising an inhibitor of a receptor.

11. A vector encoding a recombinant receptor according to any of the claims 1-8.

12. A vector encoding a prey polypeptide according to claim 9 or 10.

13. A eukaryotic cell comprising a recombinant receptor according to any of the claims 1-8.

14. A eukaryotic cell comprising a prey polypeptide according to claim 9 or 10.

15. A eukaryotic cell according to claim 13 or 14, where said cell is a mammalian cell, a fungal cell or a plant cell.

16. A kit, comprising a cloning vector allowing the construction of a vector according to claim 11 or 12.

17. Method to screen compounds that disrupt compound-compound binding using a recombinant receptor according to any of the claims 1-8 and/or a prey polypeptide according to claim 9 or 10.

18. Method to screen compounds that disrupt compound-compound binding according to claim 17, whereby said compound-compound binding is modification state dependent.

19. Method to screen compounds that disrupt compound-compound binding according to claim 18, whereby said modification is phosphorylation, acetylation, acylation, methylation, ubiquitinilation or glycosylation.

20. Method to screen compounds that disrupt compound-compound binding according to any of the claims 17-19, whereby said binding is mediated by three or more partners.

21 Method to screen compounds that disrupt compound-compound binding according to claim 20, whereby one or more of the partners is not or not completely of proteineous nature.

**Abstract**

The present invention relates to a recombinant receptor, comprising a ligand-binding domain and a signaling domain that comprises a heterologous bait polypeptide, which receptor is inactivated by binding of a prey polypeptide to said heterologous bait peptide, either in presence or absence of a ligand binding to said ligand-binding domain. The receptor is activated by addition of a compound that disrupts the bait-prey interaction. The present invention also relates to a method to screen compounds that disrupt compound-compound-binding using said recombinant receptor.

Figure 1

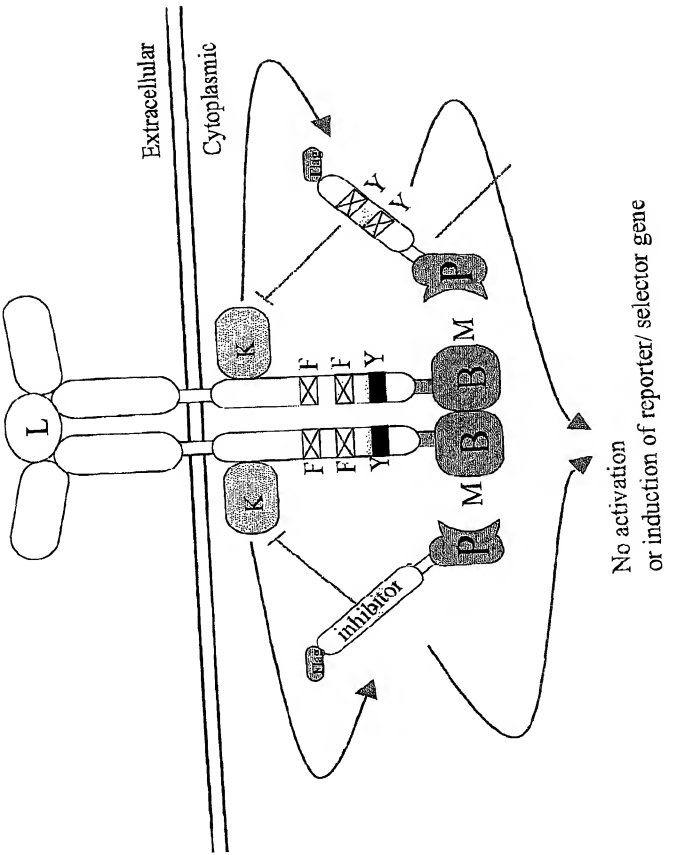
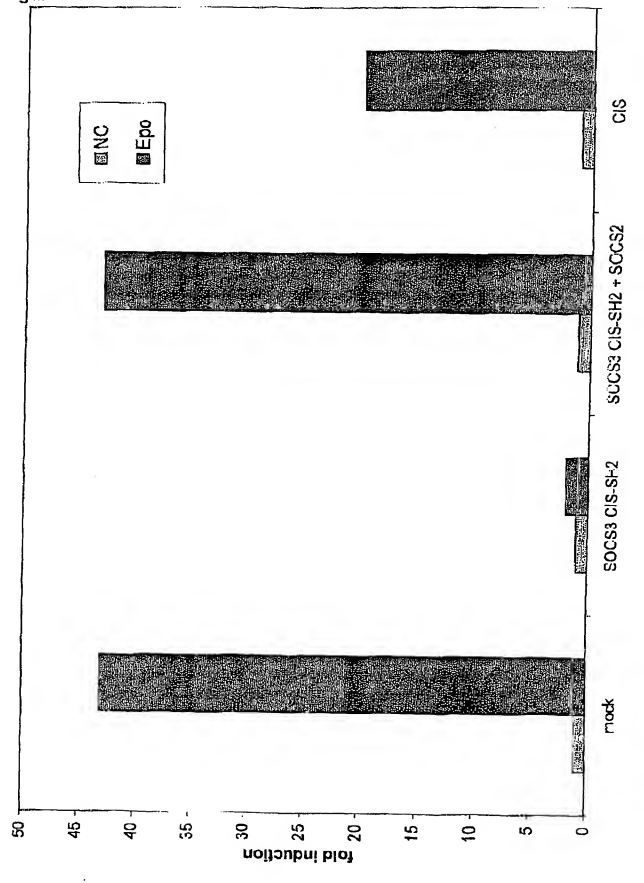

Extracellular
Cytoplasmic
L
K
K
F
F
Y
F
F
Y
B
B
M
B
M
P
P
Y
Y
inhibitor
No activation
or induction of reporter/ selector gene

Figure 2


NC
Epo
fold induction
0
5
10
15
20
25
30
35
40
45
50
mock
SOCS3 CIS-SH-2
SOCS3 CIS-SH2 + SOCS2
CIS

SEQUENCE LISTING

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<120> Reversed mammalian protein-protein interaction trap

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